

Video Article

Spatial Temporal Analysis of Fieldwise Flow in Microvasculature

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Abstract

Changes in blood flow velocity and distribution are vital in maintaining tissue and organ perfusion in response to varying cellular needs. Further, appearance of defects in microcirculation can be a primary indicator in the development of multiple pathologies. Advances in optical imaging have made intravital microscopy (IVM) a practical approach, permitting imaging at the cellular and subcellular level in live animals at high-speed over time. Yet, despite the importance of maintaining adequate tissue perfusion, spatial and temporal variability in capillary flow is seldom documented. In the standard approach, a small number of capillary segments are chosen for imaging over a limited time. To comprehensively quantify capillary flow in an unbiased way we developed Spatial Temporal Analysis of Fieldwise Flow (STAFF), a macro for FIJI open-source image analysis software. Using high-speed image sequences of full fields of blood flow within capillaries, STAFF produces images that represent motion over time called kymographs for every time interval for every vascular segment. From the kymographs STAFF calculates velocities from the distance that red blood cells move over time, and outputs the velocity data as a sequence of color-coded spatial maps for visualization and tabular output for quantitative analyses. In normal mouse livers, STAFF analyses quantified profound differences in flow velocity between pericentral and periportal regions within lobules. Even more unexpected are the differences in flow velocity seen between sinusoids that are side by side and fluctuations seen within individual vascular segments over seconds. STAFF is a powerful new tool capable of providing novel insights by enabling measurement of the complex spatiotemporal dynamics of capillary flow.

Video Link

The video component of this article can be found at <https://www.jove.com/video/60493/>

Introduction

The microvasculature plays a critical role in physiology, ensuring effective perfusion of tissues under changing conditions. Microvascular dysfunction is associated with myriad conditions including long-term cardiovascular morbidity and mortality, development of dementia, and disease of both liver and kidney and thus is a key factor of interest in a broad range of biomedical investigations^{1,2,3,4,5}. While multiple techniques have been used to evaluate tissue perfusion, only intravital microscopy enables data collection at the temporal and spatial resolution necessary to characterize blood flow at the level of individual capillaries.

Microvascular flow can be visualized in fluorescence microscopy either by the movement of fluorescent microspheres or by the movement of red blood cells against the background of membrane-impermeant fluorescent markers (e.g., fluorescently-labeled dextran or albumin)^{6,7}. Microvascular flow can be imaged in superficial cell layers using widefield microscopy, or at depth using either confocal or multiphoton microscopy. However, capillary flow rates are such that the passage of red blood cells cannot generally be captured at speeds less than 60 frames/s. Since most laser scanning confocal and multiphoton microscopes require 1–5 s to scan a full image field, this speed can generally be accomplished only by limiting the field of view, sometimes to a single scan line⁸. The process of limiting measurements to selected capillary segments (1) has the potential to introduce selection bias and (2) makes it impossible to capture spatial and temporal heterogeneity in the rates of capillary blood flow. In contrast, images of capillary networks can be collected at speeds exceeding 100 fps using widefield digital microscopes equipped with scientific complementary metal oxide semiconductor (sCMOS) cameras^{9,10}. These inexpensive systems, common in typical biomedical laboratories make it possible to image microvascular flow across entire two-dimensional networks, essentially continuously. The problem then becomes one of finding an analysis approach that is capable of extracting meaningful quantitative data from the massive and complex image datasets generated by high-speed video microscopy.

To enable analysis of full-field flow data we have developed STAFF, novel image analysis software that can continuously measure microvascular flow throughout entire microscope fields of image series collected at high speed¹¹. The approach is compatible with a variety of different experimental systems and imaging modalities and the STAFF image analysis software is implemented as a macro toolset for the FIJI implementation of ImageJ¹². The underlying principle used here to visualize microvascular flow is that first, some contrast must be provided to be able to image the red blood cells within capillaries. In our studies, contrast is provided by a bulk fluorescent probe that is excluded by the red blood cells. The velocity of flow can then be quantified from the displacement of the red blood cells that appear as a negative stain within the fluorescently labeled plasma in images collected at high speed from a living animal⁸. We then use STAFF to make plots of distance along each capillary segment over multiple intervals of time called kymographs, then detect the slopes present in the kymographs¹³, and from those slopes calculate the rates of microvascular flow. The approach can be applied to images collected from any capillary bed that can be accessed for imaging. Here we describe the application of IVM and STAFF to studies of blood flow in the liver.

Protocol

All animal experiments were approved and conducted according to the Institutional Animal Care and Use Committee guidelines of Indiana University, and adhered to the NRC guide for the care and use of animals.

1. Surgical Preparation for Intravital Microscopy

NOTE: This is not a survival surgery. Once section 1 "Surgical preparation for intravital microscopy" is begun, work cannot be paused until the completion of section 2 "Intravital microscopy".

1. Acclimatize 9–10 week-old male C57BL/6 mice, for at least 4 days and fast for 16 h prior to studies.
2. Weigh the animal, sedate with 5% isoflurane and place on a heating pad to maintain body temperature. Use an oxygen flow rate of 1–2 L/min and maintain sedation with 1–2% isoflurane. Check reflexes by toe pinch. Monitor temperature using a rectal thermometer. Monitor heart rate and respiration visually.
3. When anesthesia is stable, shave the area for jugular cannulation placement and the area below the rib cage for the exposure of the liver.
4. **For jugular cannulation, make a 1 cm left ventral incision 1–2 cm below the mouse's jaw. Clear away all fat and fascia surrounding the jugular vein. Tie off the anterior end of the jugular using 06 suture string to prevent bleeding.**
 1. Make a tiny nick in the jugular vein and slide the cannula (30 G x ½ in, needle), needle holder and polyethylene tubing (0.011 x 0.024 inches² attached to a Luer stub adapter and filled with 0.9% saline) in about 1 cm, and secure at the posterior end of the jugular using 0-6 suture string.
5. Using the jugular cannula deliver 70 kDa fluorescein dextran (to a dose of 30 mg/kg via injection of 0.1 mL of a 9 mg/mL solution in saline).
6. Expose the liver for imaging by making a 4–6 cm incision across the torso, 1–2 cm below the middle of the rib cage.
7. Place a wet (soaked in 0.9% saline) 2 x 2 inches² gauze sponge below the left lateral liver lobe. Place tape on the periphery of the glass window of a 40 mm coverslip-bottomed dish and apply cyanoacrylate adhesive to the tape. Press the glass plate to the liver and using cotton tipped applicators press the gauze into the glue on the tape to minimize tissue motion for microscopy.
8. Move animal to the microscope stage. Add sterile 0.9% saline into the coverslip-bottomed dish to keep the liver moist throughout the imaging session. Maintain temperature at 36–37 °C via heating pads mounted on the stage, a heating pad placed over the animal and an objective heater.

2. Intravital Microscopy

1. Perform intravital microscopy on an inverted epifluorescence microscope equipped with a video camera capable of high-speed image capture.
NOTE: Here, a Xenon arc lamp, fluorescein-specific excitation (480–500 nm) and emission (507–543 nm) filters, Plan Fluor 20x, N.A. 0.75 water immersion objective, and a high-speed sCMOS camera were used.
2. Select the area of interest for capillary blood flow analysis using minimal illumination.
3. Set the exposure time short enough for the camera to be able to acquire 100 fps. Set illumination level to clearly visualize red blood cell shadows and the topology of the vasculature while also avoiding phototoxicity and photobleaching of the fluorescent probe.
4. Collect images at a rate of 100 fps. Set the camera pixel resolution between ~0.65 and ~1.3 µm/pixel. Set frame size between 512 x 512 and 1024 x 1024 pixels. Set bit depth between 8 and 16 bits. Use the highest resolution, frame size and bit depth that still allows 100 fps image collection on the system.
5. Save time series file(s) as a sequence of TIF files or as native camera/microscope format if Bio-Formats¹⁴ in FIJI can open the native format files.
6. Image multiple areas over time. Maintain the mouse on the microscope stage for up to 2 h. Euthanize the mouse at the end of imaging.
NOTE: The duration of the time series will be based upon balancing the need to image for an interval of time that embraces the variability, while not affecting the viability of the tissue. The duration of the time series may also be dictated by file size considerations; a 1 min time series of 1024 x 1024 pixel 16-bit images collected at 100 fps will generate a file that is 12 GB in size. File size considerations may also dictate frame size and bit depth used. There are no explicit size limits within STAFF.

3. Define the Vascular Network Using TrakEM2 in FIJI

NOTE: The protocol can be paused after saving work at any point in section 3.

1. Download and install FIJI from <https://imagej.net/Fiji/Downloads>.
NOTE: Version 1.51n of Fiji was used for this project, and can be downloaded from <https://downloads.imagej.net/fiji/Life-Line/fiji-win64-20170530.zip>. Note that on Windows, FIJI should be installed in the user space and not in Program Files.

- Open the movie file in FIJI using **File > Open** or using **File > Import > Image sequence**. Select a single image from the stable images between respirations where the topology of the vascular network is easy to see. Select **Image > Duplicate** and duplicate the selected single image (not the stack) and save the image in a new folder. Do not include spaces in file or folder names.
- Set up a new blank TrakEM2 project by selecting **File > New > TrakEM2 (blank)**. Select the new folder containing the single image from the movie as the project folder. The TrakEM2 windows will open.
- Right-click in the main work area and select **Import > Import Image**. Navigate to the single image saved in step 3.3 and select it.
- Right-click in the main work area again and select **Display > Autoresize canvas/Layer set**. Left-click in the main work window. The image will fill the work area.
- To select the areas in the image that contain the vascular network, setup area list selection in TrakEM2. In the smaller TrakEM2 window (Template, Project Objects, Layers) right-click on **"• anything"**. Select **Add new child > area list**.
- In the smaller TrakEM2 window, drag **"Template > anything"** onto **"Project Objects > project"**. Then drag **"Template > anything > • area list"** onto **"Project Objects > project > anything"**. Under **"Project Objects > project > anything"**, **• area list** will now be present.
- In the main TrakEM2 window under the **Z space tab**, a bar labeled area list was created. Click to select it. In the main TrakEM2 window also select the **paintbrush tool**. Press **Shift** and roll the mouse wheel to select an appropriate size for the paintbrush, e.g., smaller than the diameter of the vasculature. To save the TrakEM2 setup press **Control + S** keys.
- Paint the vascular network using the **paintbrush tool**. To erase hold down **Alt** while using the paintbrush tool. Do not include out-of-focus regions. Save often using **Control + S**.
- When labeling of the vascular network is complete, right-click in the main TrakEM2 window and select **Export > AreaLists as labels** (tif). In the popup window select **Scale 100%** and **Export All area list**. Close TrakEM2 windows and choose **yes** for **Save project**.
- The image of AreaLists will open and may appear as a blank black image. In the main FIJI Menu select **Image > Adjust > Brightness/Contrast**. In the B&C window press the **auto button** and the AreaList will become visible. In the main FIJI menu select **Image > Lookup Tables > Invert LUT**. Save this image of black labels on white background and close the image.
- Open the labels file in FIJI. Select **Plugins > Skeleton > Skeletonize**. Save the skeletonized image as a tif. Use the skeleton.tif file as one of the input files needed to run the STAFF flow analysis. When saving the file, do not use spaces in file name.
- Manually edit the skeleton file as needed, by placing a gap (using the **paint tool**) in the line drawing for example at locations where branches in the vasculature have not been captured because they go out of the image plane.

4. Prepare the Movie Sequence for STAFF Analysis

- Open the movie file in FIJI using **File > Open** or **File > Import > Image sequence**. Select a time period of the image sequence for flow analysis (can be seconds to minutes, hundreds to tens of thousands of frames) where tissue position has remained stable over time, except for during respiration.
- Select **Image > Duplicate** and duplicate the selected portion of the sequence by typing in the numbers of the beginning and end frames. Check image spatial and temporal calibration under **Image > Properties** and correct if necessary. Save the image file as a TIF (or as an uncompressed AVI). This movie.tif is needed as an input file to run STAFF analysis. Do not include spaces in file name.

5. Install STAFF Macros into FIJI

NOTE: (Important) Multiple folders within the FIJI folder subdirectories have similar file names, some capitalized, some not. Be certain that the correct folders are selected when installing STAFF.

- Download the STAFF macros from <https://github.com/icbm-iupui/STAFF>.
- To install, first open the FIJI folder.**
 - On Windows find the folder where FIJI is installed in the user space, most commonly on the desktop. On MacOS find the FIJI icon in the Applications folder, right click and select **Open**.
- In the FIJI folder, open the plugins folder. Inside the plugins folder, open the Macros folder. Copy STAFF.ijm into this Macros folder: Fiji\plugins\Macros\STAFF.ijm.
- In the FIJI folder, open the plugins folder. Copy STAFF_Dir.jar into this plugins folder: Fiji\plugins\STAFF_Dir.jar.
- In the FIJI folder, open the macros folder. Inside this macros folder, open the AutoRun folder. Copy STAFF_Loader.ijm into this AutoRun folder: Fiji\macros\AutoRun\STAFF_Loader.ijm.
- Start FIJI and verify macro installation. To verify installation, select **Plugins > Macros**. When correctly installed the dropdown menu will include: Open-Create Project, Analyze Skeleton, Edit Time Intervals, Analyze Flow and Produce Spatial Map.
- If these commands are not present in the Macros menu, select **Plugins > Macros > Install**, open the Fiji\plugins\Macros folder, select the STAFF.ijm file, then click the **OK** button. The commands should now appear in the **Plugins > Macros** menu.

6. Quantifying Vascular Flow Using STAFF

- Create a new project.**
 - Select **Plugins > Macros > Open-Create Project** and follow prompts to create or update a configuration file.
 - From the **Open-Create Project** menu, navigate to and select the Project Directory, Input File Folder and the input files Movie.tif and Skeleton.tif. Edit the auto-populated output file names if necessary.
 - Input values for shortest segment length (20 μm), max speed measured (2,000 $\mu\text{m/s}$) and max speed mapped (1,000 $\mu\text{m/s}$).
NOTE: The product of shortest segment length and frames per second gives the max flow speed that can theoretically be measured. The highest capillary flow speeds reported in literature are around 2,000 $\mu\text{m/s}$ ¹⁵. In the liver, we observed that capillary flow speeds generally averaged around 300 $\mu\text{m/s}$ and rarely exceeded 1,000 $\mu\text{m/s}$.
 - Type in the values for pixel size and frame rate for the image sequence (from image metadata or experimental notes). These values require user input. Check the **Flicker Correction** box if the images have periodic background intensity flicker.

5. Select parameters for best visualization of the data. Select max speed mapped to include about 95% of the data so that the data is mapped across the full range of the color scale. STAFF maps high speed outliers to the high-speed end of the color scale.
2. **Analyze the skeleton.**
 1. Select **Plugins > Macros > Analyze Skeleton**. The skeleton.tif file will open and the Region of Interest (ROI) Manager will open and run.
NOTE: Analyze Skeleton classifies each pixel by its number of neighbors as either at an endpoint, at a junction, or within a segment. For each segment an identifying number (ID number), a calibrated branch length and the spatial coordinates of the branch ends are recorded.
 2. Wait for segment IDs to appear on the skeleton, the segment list to appear in the ROI manager, and a popup indicating that ROI Manager file for the skeleton is saved. Click **OK**.
 3. Verify that between respiration movements, the skeleton remains over where red blood cells pass through the capillary (e.g., not the edge and not outside the capillary). Display the labeled segments as an overlay on the movie, by opening the movie file, then dragging the ROI zip file onto the open movie. Play the movie with the segments overlaid.
3. **Select time intervals.**
 1. Select **Plugins > Macros > Select Time Intervals**. Wait while the macro generates a kymograph from a single segment over the total time for the movie. From this kymograph, the user selects time intervals for analysis. Use **Control + +** keys to increase the size of the kymograph if needed.
 2. The rectangle selection tool is automatically activated. Draw a rectangle around each time interval. A good starting point for time interval length is the 1–2 s interval between respirations, which are apparent as horizontal blurred regions in the kymograph.
 3. Click the **T** key or the **Add** button in the ROI manager to record time interval selections. Repeat for as many time intervals as desired. Make selections sequentially from the top (or left) to bottom (or right) of the kymograph.
 4. Evaluate parameter choices (from Open-Create Project) by selecting a small number of time intervals (3 to 4) and completing analysis for just those intervals, since Analyze Flow (the next step) can take hours for large datasets.
 5. Click **OK** in the **Make an ROI** for each time interval window when done. A popup window appears when the selected intervals have been saved in the **Project Folder**. Click **OK**.
4. **Analyze flow.**
 1. Select **Plugins > Macros > Analyze Flow** and the **Analyze Flow Parameters** dialogue box opens and displays the values entered in the Open-Create Project step. Edit if needed then click **OK**.
 2. The **Output File Names** dialogue box opens and displays the names entered in the Open-Create Project step. Edit if needed then click **OK**.
 3. A dialogue box opens asking if the user is ready to begin analysis. Click **OK** and the analysis will begin. Wait for a dialog box that opens to indicate when Flow Analysis is complete.
NOTE: Time needed depends on processor speed and size of the dataset.
 4. Confirm that results files have been stored as .csv format spreadsheet files in the Project Folder.
NOTE: Output files include: *segment_velocities.csv* contains velocity values that have positive and negative values indicating flow direction. Cells representing segments less than the minimum length contain the text SHORT. Cells with velocity values greater than the maximum measured speed contain the text OUT (for outlier). *kym_ang.csv* contains kymograph angles measured by the Directionality plugin in FIJI. *good_fit.csv* contains goodness of fit of a curve to the distribution of angles measured from each kymograph. Poor goodness of fit (< 0.8) can indicate hidden branch points in a segment, change in velocity during the time interval, lamp or room light flicker.
 5. Check to verify that *segment_velocities.csv* contains few OUT values. If there are a large number of OUT values, verify that the minimum segment length to analyze is not less than 15–20 μm , then rerun **Analyze Flow**. If the new *segment_velocities.csv* still contains a large proportion of OUT values, then check the **flicker correction** box, then rerun **Analyze Flow**.
5. **Produce spatial maps.**
 1. Select **Plugins > Macros > Produce Spatial Map** and the **Spatial Map Parameters** window will open, displaying the values entered in the Open-Create Project step. Edit if needed, or to continue select **OK**.
 2. Watch as a temporal sequence of spatial maps of flow velocities is generated with color indicating flow speed. The output is in the form of a .tif image stack with one image for each time interval. Scroll through the .tif stack to visualize spatial and temporal variation in flow. Save the stack as an AVI file (uncompressed for maximum portability) to share as a movie.

7. Quantitative Analysis Using STAFF Output

1. Open the *segment_velocities.csv* file in a spreadsheet or statistical analysis program. These values have positive or negative sign that indicates direction of flow relative to the start/stop points of that segment (recorded in the Segment ROI file).
2. Make a new spreadsheet page containing the absolute values of all the velocity values. Use these values to calculate overall average flow velocity, average flow velocity for all vascular segments over time, flow velocity for each vascular segment over time, and make histograms of velocity distributions.
3. Calculate values around specific morphological regions, or other regions of interest by first finding the specific relevant segments in the labeled skeleton and then performing analyses on these selected segments.

Representative Results

STAFF analysis generates a complete census of microvascular velocities across entire microscope fields over periods of time extending from seconds to minutes. Representative results are presented in **Figure 1**, **Figure 2**, **Figure 3**, and **Figure 4**. **Figure 1** shows an example

of a time series of the microvascular network in the liver of a mouse, the generation of the skeletonized image that is used to define the axis of microvascular flow, and the STAFF-generated map of individual vascular segments identified for quantification. STAFF then uses the skeletonized image to break the microvascular network down into individual segments, then generates images of kymographs for each segment. These images are provided to the user, along with tools to identify the time intervals to be used for kymograph analysis (**Figure 2**). STAFF then uses the skeleton, and the user-supplied time intervals to break the kymograph of each segment into individual segment-time intervals. STAFF then identifies the predominant angle in the kymograph from each segment-time interval and provides velocity measurements as .csv data files (**Figure 3**) and in the form of stacks of color-coded velocity map images (**Figure 4**). In order to support exploration of the data analysis pipeline, STAFF also provides .csv files containing all kymograph angle measurements and goodness-of-fit values.

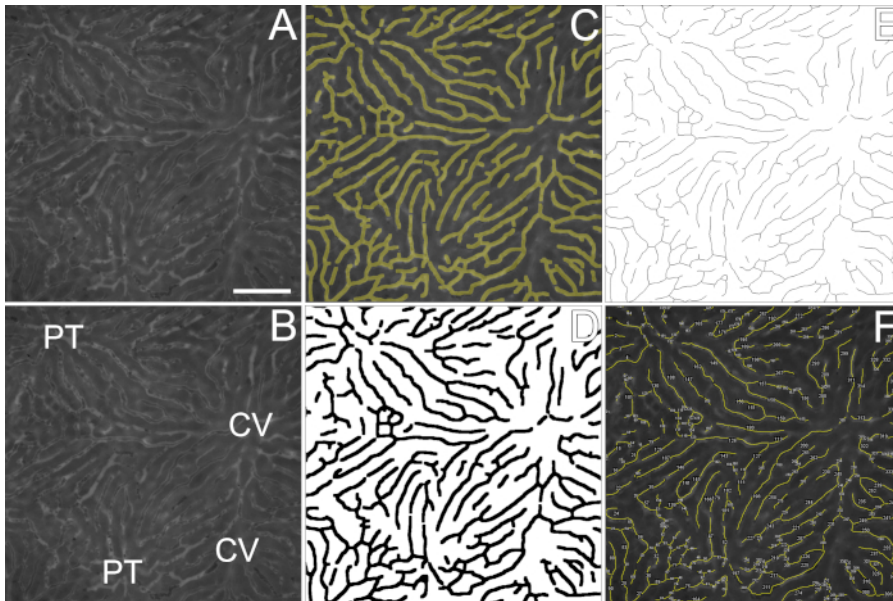


Figure 1: Generating the vascular skeleton. (A) Original image of a single frame from time series images collected from the liver of a living mouse. Scale bar = 100 μm . (B) Image shown in Panel A with central veins (CV) and portal triads (PT) indicated, to identify main directions of sinusoid flow. (C) Image from Panel A with overlay of TrakEM2 segmentation of sinusoids. (D) Binary image of TrakEM2 segmentation. (E) Skeletonization of TrakEM2 segmentation. (F) STAFF output image of individual vascular segments with labels. [Please click here to view a larger version of this figure.](#)

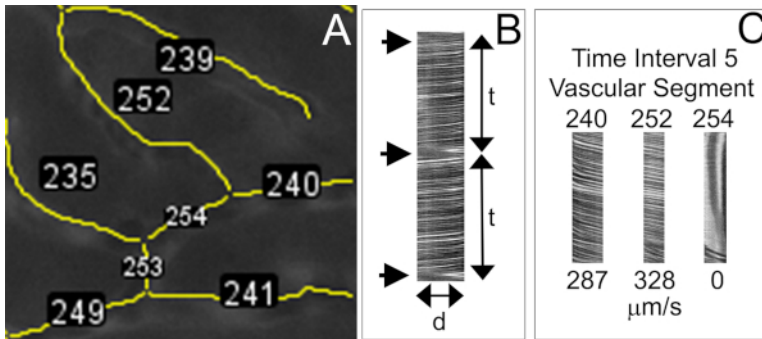


Figure 2: Kymograph analysis. (A) Magnified image of vascular segments shown in **Figure 1F**. (B) Typical kymograph from one segment collected over two inter-respiratory time intervals. Periods of respiration are noted with arrows. (C) Kymographs for segments 240, 252 and 254 from panel A. [Please click here to view a larger version of this figure.](#)

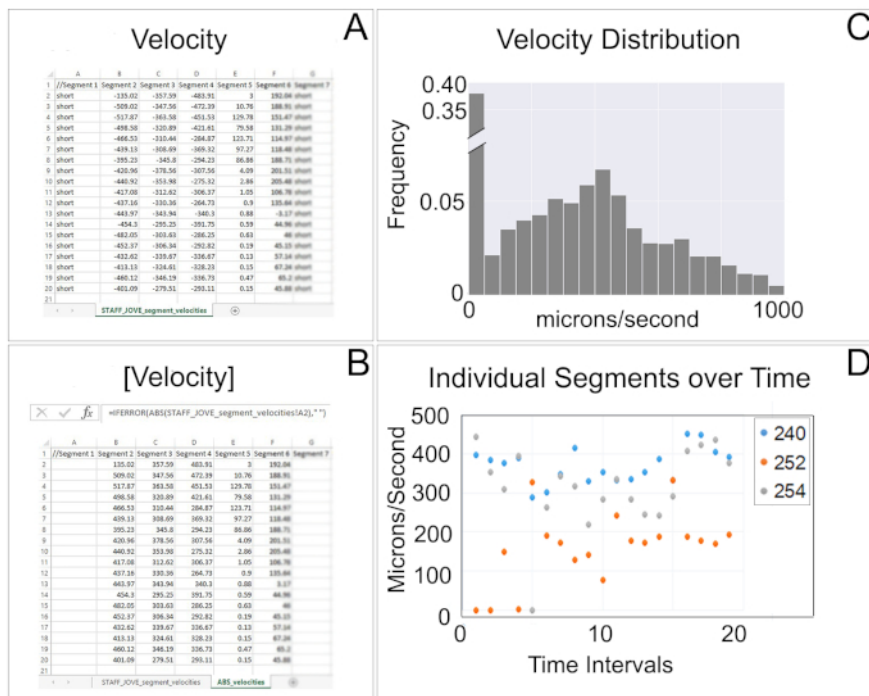
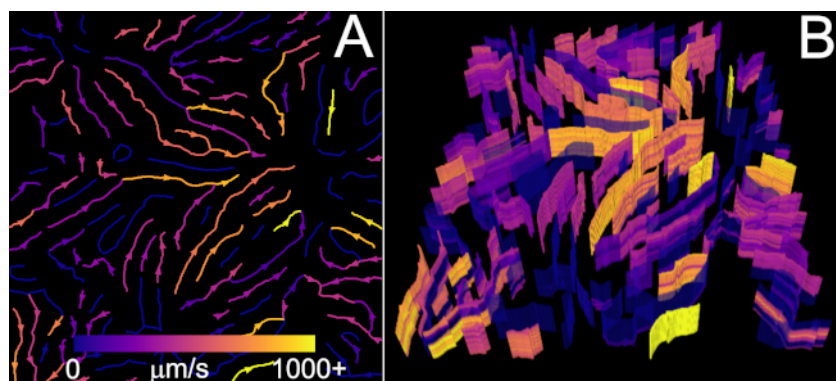


Figure 3: Velocity data analysis. (A, B) Tables of velocity measurements of four segments over 19 time intervals expressed either as velocity with direction (A) or as absolute velocity (B). (C) Histogram showing distribution of absolute velocity values across the entire field over the entire 20 s time period. (D) Graph of the velocities of the three segments whose kymographs are shown in Figure 2C over the entire 20 s period. Please click here to view a larger version of this figure.



Discussion

There are multiple critical steps in this protocol. First, minimization of motion during intravital imaging of the liver is essential for generating movies that are usable for capillary flow analysis using STAFF. Due to the proximity of the diaphragm, short periods of respiration-induced motion occur, with the secured liver returning to its initial position after each breath. Securing the surgically exposed liver against the coverslip-bottomed dish using gauze, then imaging from below using an inverted microscope serves to immobilize the organ between respirations^{16,17,18,19}. Second, an image acquisition speed of 100 fps is strongly recommended because the speed of flow that can be measured is a function of the speed of image acquisition and the minimum length of the segments in which flow is measured¹¹. Third, producing a high-quality skeleton, the line drawing representation of the vascular network, is the next critical step in obtaining capillary flow velocities using STAFF. Skeleton line segments should lie near the midpoint of the vasculature between respirations over the time course being analyzed and vascular branching out of the image plane should be identified. The locations of these hidden branches along a vascular segment can be inferred by viewing the movie, by examining the kymograph or examining the velocity values over time for that segment. Viewing the movie, these vascular segments are seen either as having bidirectional flow, emanating away from or converging towards the location of the unseen branch, or as having an abrupt change in flow speed at that point along the vascular segment. The location of hidden branch points can be identified in kymographs as the location of a change in angle that is produced by the change in flow direction or velocity. In the spreadsheet, vascular segments with hidden branch locations may spuriously change direction (sign) or change between the values of the two contributing segments. If the time series includes too many branch points that

are out of the image plane, STAFF analysis should be repeated after manually editing the skeleton, by placing a gap (using the paint tool) in the line drawing at the locations of the missed branch points.

We uncovered a common issue in image acquisition that typically goes unnoticed but had a strong effect on measuring flow velocities using STAFF. In image acquisition, instability or “flicker” in the intensity of the epi-illumination lamp light source or from area lighting in the microscope room can occur. From either source, light/dark banding over time with the period of the flicker occurs at zero angle in the kymographs. If the zero angle peak is the major peak, then even if the angle produced by motion of red blood corpuscles through the vessel where flow speed is being measured is obvious to the human eye, the directionality plugin fits a curve to the zero peak and reports a value very near zero degrees, resulting in velocity measurements that are unrealistically high. Even if the zero-angle peak is not the major peak, it will influence the directionality curve fit such that the velocity reported is shifted toward a higher value. To address this problem, STAFF provides a “Flicker correction” option that ignores peak angles occurring at 0°. This modification eliminated the effects of lamp flicker without affecting velocity quantifications.

The main limitation of obtaining flow velocities using widefield microscopy is that image acquisition is restricted to thin preparations such as mesenteric vasculature, or the zebrafish intersegmental vessels or the superficial layers of organs such as liver or kidney that can be exteriorized.

A significant advantage of using STAFF over existing methods of flow quantification is that it enables rapid and unbiased detection of spatial and temporal patterns of vascular flow. Collection and analysis of microvascular flow data using raster scanning systems are generally limited to measurements of single user selected capillaries at a time^{20,21,22,23}. Methods exist to extract flow velocities across fields^{24,25,26}, however none of these approaches support analysis across entire fields and all are labor intensive. Using STAFF, analysis of every capillary segment over time across the entire image field of a dataset with tens of thousands of images can be accomplished within a day. Manual analysis of even a single dataset of full-field vascular flow would take months to years. Thus, manual analysis is impractical even for characterization of normal flow and clearly does not permit comparison of multiple treatment groups.

The ease of STAFF quantification of spatiotemporal patterning of vascular flow provides the opportunity for future users to link vascular morphological observations to effects on capillary flow velocity patterning. By defining the relationship between the vascular morphology measures of vessel diameters and network topology to flow velocity patterning we may then be able to predict flow patterns from vascular morphology. Similarly, correlating vascular flow patterning and vascular morphology with events such as timing, localization and extent of immune cell infiltration, tissue damage from toxicant exposure or disease, or status of intracellular transport, would not only give us a better understanding of flow patterning and cellular function in health and disease, but would also provide a framework for identifying particularly harmful pathophysiological scenarios.

Disclosures

The authors report no competing interests.

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